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14. ABSTRACT

The purpose of this project is to determine how changes in levels of Zn2+ affect the occupancy of proteins that include Zn2+ finger motifs and how in turn Zn2+ occupancy affects the function of prostate cancer cells and in particular response to chemotherapy. Our findings show that prostate cancer cells are highly sensitive to perturbations in Zn2+ concentration with both increases and decreases of Zn2+ causing cytotoxicity and increasing sensitivity to chemotherapy. Our studies showed that the effects of exogenous Zn2+ on prostate cancer viability were reversible and that cells adapted to changes in Zn2+ levels. In contrast, Zn2+ chelation was irreversible and led to apoptosis. For the chemotherapy drug-candidate F10 Zn2+ chelation was synergistic and has maximal effect when administered more than 24 h after treatment. A novel finding was that the serine protease Omi/HtrA2 enhanced F10-induced apoptosis in a Zn2+-dependent manner Treatment with F10 induced p53-responsive genes p21 and Bax in a Zn2+-dependent manner. Extensive molecular dynamics simulations were undertaken on the Zn2+-finger peptide from NEMO, a component of the NF-kB responsive pathway. These computational experiments revealed the free energy for Zn2+ dissociation from this peptide was ~2.5 kcal/mol. These experimental and computational studies will be pursued in future grant applications to develop a comprehensive understanding of the effects of Zn2+ levels on the response of prostate cancer cells to chemotherapy.

15. SUBJECT TERMS
Prostate cancer, Zn2+, metal-protein interactions, structural biology

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INTRODUCTION: The project "Metal Occupancy of Zinc Finger Motifs as Determinants for Zn2+-Mediated Chemosensitization of Prostate Cancer Cells" provides information on the extent that specific types of Zn2+-binding motifs are perturbed in prostate cancer cells in response to treatment with exogenous Zn2+ or chelators of Zn2+. The project is collaborative and is directed overall by Dr. Gmeiner in the Department of Cancer Biology at Wake Forest School of Medicine in collaboration with Dr. Salsbury in the Department of Physics at Wake Forest University. Dr. Gmeiner's laboratory is performing cell-based assays evaluating the effects of exogenous Zn2+ as well as chelation of Zn2+ on prostate cancer cell viability and induction of apoptosis. Dr. Salsbury's laboratory is performing computational studies investigating the stability of interactions between Zn2+-binding motifs in proteins and Zn2+ ions. This project seeks to identify the structure/function relationships in Zn2+-binding proteins that are responsible for the anti-tumor properties of Zn2+ and Zn2+-chelators. We expect the work completed to date to result in at least two publications the first of which is included in draft form (Gmeiner, et al.; **appendix #1**) and the second as a report summary (Godwin et al., 2013; **appendix #2**). This project has contributed to two new letters of intent (LOI) to the DoD PCRP – one to the Idea Award mechanism "Zn2+ Modulation for Treatment of Advanced Prostate Cancer" (Gmeiner PI) and the second to the Synergistic Idea award mechanism "Omi/HtrA2 as a Novel Target for Prostate Cancer Treatment" (Gmeiner & Salsbury co-PI's). The Idea award LOI was invited for a full proposal and is under review.

BODY:

Aim 1: Determine to what extent Zn2+ increases chemotherapy-induced apoptosis in LNCaP and C4-2 cells in part by prolonging the half-life of p53 and increasing the levels of p53-induced pro-apoptotic target genes including Bax and PUMA.

Task 1A: Quantify intracellular Zn2+ levels in LNCaP, C4-2, and PC3 cells cultured under low, moderate, and high Zn2+ conditions using Fluo-Zn with fluorescence detection.

We developed fluorescence-based assays using 96 well plates and also used confocal microscopy to measure relative levels of intracellular Zn2+ and changes in intracellular Zn2+ concentrations in PC3 and C4-2 prostate cancer cells in response to treatment with either exogenous Zn2+ or Zn2+-chelators. These studies are included in the manuscript in preparation (**Appendix #1**; Figure 2; references 1-7) as well as in Figure 1.

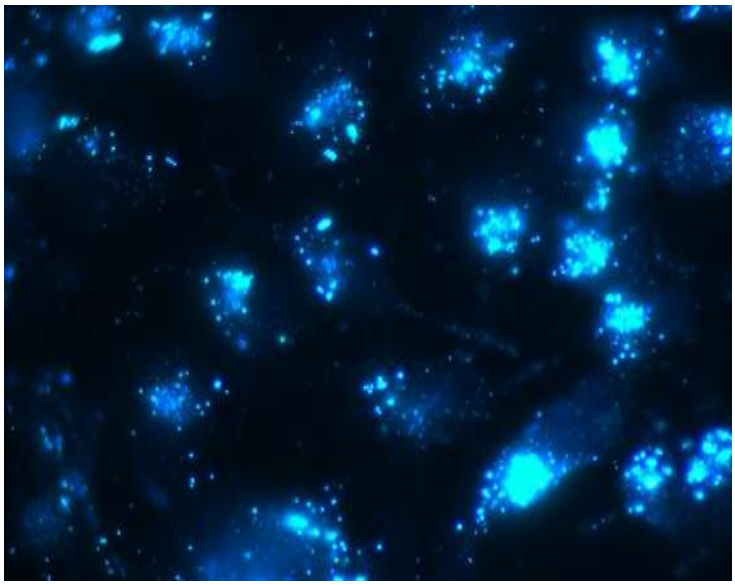

Figure 1: Confocal microscopy analysis of intracellular Zn2+ levels in C4-2 cells measured using Zinquin in cells treated with 6 micromolar Zn2+-pyrithione (ZnP). Fluorescence significantly above background is observed. For quantification and statistical analysis see appendix #1.

Task 1B: Evaluate apoptosis induction in LNCaP and C4-2 cells (both wt-p53) and PC3 (p53-null) cells following treatment with docetaxel using a caspase-glo 3/7 assay kit. Cells will be grown under low, moderate, and high Zn2+ conditions. (months 1-2).

We have completed these studies in PC3 and C4-2 cells using the novel fluoropyrimidine chemotherapy drug-candidate F10 as the chemotherapy agent. A summary of these studies is included in the manuscript in preparation (**Appendix #1**; Figure 4) and data for F10 in combination with the Zn2+-chelator TPEN is included in Figure 2. Our studies demonstrated that both exogenous Zn2+ and Zn2+-chelation is cytotoxic to prostate cancer cells and that Zn2+ chelation was highly effective at increasing the efficacy of chemotherapy.

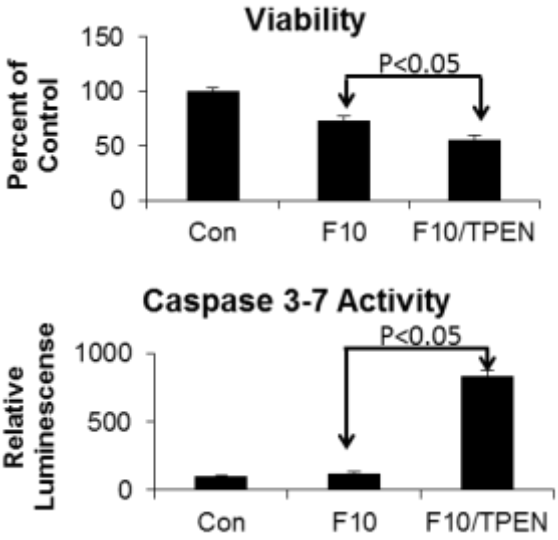

Figure 2. Effects of decreasing Zn2+ levels in C4-2 prostate cancer cells using the chelator TPEN in combination with the chemotherapy drug-candidate F10. Effects on cell viability are shown in the upper panel and effects on caspase activation and induction of apoptosis are shown in the lower panel.

The substantial enhancement in apoptosis induction in prostate cancer cells in response to Zn2+-chelation with TPEN in combination with chemotherapy resulted in our developing a new project "Zn2+ Modulation for Treatment of Advanced Prostate Cancer". We submitted a letter of intent (LOI) for the PCRP Idea mechanism and were invited to submit a full application that is currently under review.

Task 1C: Evaluate stabilization of p53 in LNCaP and C4-2 cells cultured under low-, moderate-, or high Zn2+ conditions in response to treatment with docetaxel.

For these studies we have focused mainly on evaluating expression of p53-target genes rather than levels of p53 itself as these endpoints are most relevant for induction of apoptosis (see Task 1D).

Task 1D: Evaluate expression of p53 target genes (e.g. PUMA, Bax, Mdm2) in LNCaP and C4-2 cells under low, moderate, and high Zn2+ conditions in response to treatment with docetaxel.

We have completed studies evaluating how modulation of intracellular Zn2+ levels using the Zn2+-chelator TPEN affects expression of p53 target genes including Bax and PUMA in C4-2 prostate cancer cells. A representative Western blot is included in Figure 3.

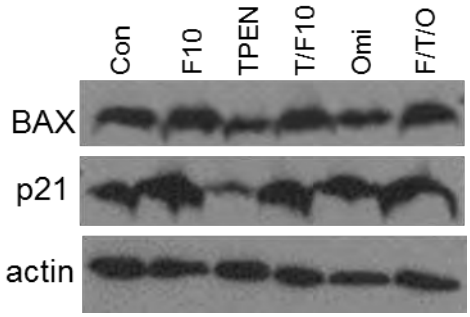

Figure 3. Representative Western blot evaluating expression of two p53-responsive genes: p21 (left) and Bax (right) in response to treatment of C4-2 cells with lane 1: no treatment; lane 2 F10; lane 3 TPEN; lane 4 F10+TPEN; lane 5 inhibitor of Omi HtrA2; lane 6 F10+TPEN+Omi. The pro-apoptotic p53 gene Bax was notably upregulated in response to treatment with chemotherapy in combination with Zn2+-chelation.

An interesting and unexpected finding in these studies was that inhibition of the serine protease Omi/HtrA2 enhanced chemotherapy-induced apoptosis in prostate cancer cells in a Zn2+-dependent manner. The rationale for why we initiatied investigation of Omi/HtrA2 as a mediator of Zn2+-effects in prostate cancer cells is described in the introduction to the manuscript in preparation (**Appendix #1**). These findings served as the basis for a new Synergistic Idea application to the DoD PCRP program that was not selected for advancement although we think this a is a highly significant finding and plan to pursue it as a topic for future grant submission.

Aim 2: Determine to what extent the Zn2+-finger from p53 is in apo-form in prostate cancer cells and evaluate changes in the apo/holo ratio for Zn2+-finger peptides upon growth under low-, moderate-, or high-Zn2+ conditions.

Task 2A: Immunoprecipitate p53 from LNCaP cells treated with docetaxel cultured under low-, moderate-, or high-Zn2+ conditions and provide the immunoprecipitated protein to Dr. Javier Seravalli for ICP-MS analysis.

We met with Dr. C. Furdui to discuss immunoprecipitation procedures and made plans to initiate these studies however successful implantation requires identifying conditions in which intracellular Zn2+ levels perturb the biological activity of p53. These studies were undertaken as part of specific aim 1 and we now have a firm understanding of how Zn2+ perturbs the biological activity of p53 and p53-responsive genes however the project terminated before we could undertake the immunoprecipitation studies.

Task 2B: Culture LCaP cells under low-, moderate-, or high-Zn2+ conditions with Zn2+ enriched in 65Zn and determine the amount of Zn2+ in immunoprecipitated p53 by gamma counting.

We have not yet undertaken studies with 65Zn.

Aim 3: Evaluate the extent to which the apo-form for Zn2+-finger motifs in p53 and Mdm2 differ in structure and stability relative to the metallated structure.

Task 3A: Evaluate the conformational dynamics of apo- and holo-forms of p53 and Mdm2.

<u>Milestones/deliverables for Task3A:</u>
i) Have 16 20ns or longer simulations (four for each of two proteins in each of two states)
ii) Have detailed comparisons of apo- and holo-forms for each protein.

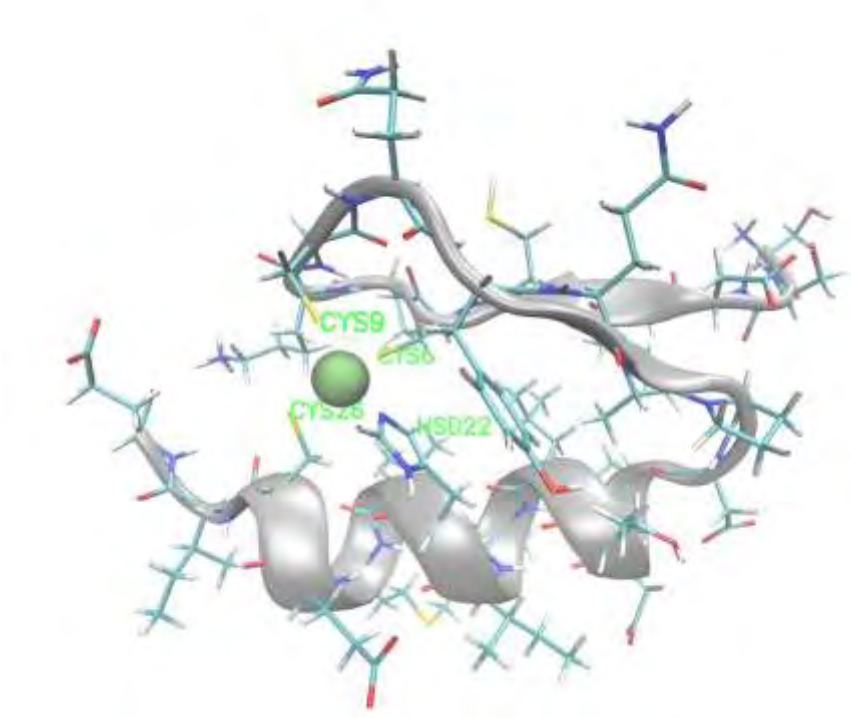

Figure 4. Structure of the Zn2+ finger from the protein NEMO with Zn2+ bound. Extensive MD simulation were run on this structure in the presence and absence of Zn2+ and energetics of Zn2+ binding were evaluated computationally.

We have completed multiple molecular dynamics simulations for the Zn2+ finger of NEMO, a protein important for transmitting signals of DNA damage from the nucleus to the cytoplasm (reference 8) and modulating the initiation of apoptosis. A summary of the MD simulations is included as Appendix #2 and we are working on preparing a manuscript based on our results. We see distinct difference in structure and energy for the apo- and holo-forms of this protein.

Task 3B: Evaluate the conformational free-energy changes that occur upon zinc binding.
Milestones/deliverables for Task 3B:
i) Have specific predictions for free-energy changes that occur due to the binding of zinc.

We have completed these studies and performed preliminary analyses. These studies indicate the free enrgy for Zn2+ binding is ~1.5 kcal/mol. A summary of these results is included as Appendix #2.

KEY RESEARCH ACCOMPLISHMENTS:

- We developed fluorescence assays for evaluating changes in intracellular Zn2+ in prostate cancer cells in response to treatment with Zn2+ and Zn2+ chelators
- We showed that the effects of chemotherapy are modulated both by exogenous Zn2+ and especially Zn2+ chelation (appendix #1)
- We showed increased expression of p53-responsive pro-apoptotic genes in response to Zn2+-chelation in combination with chemotherapy.
- We identified Omi/HtrA2 inhibition as a new strategy for prostate cancer treatment and enhancing the effectiveness of chemotherapy for prostate cancer treatment.
- We demonstrated that Zn2+ binding alters the stability of NEMO and calculated the energetic for this interaction. (appendix #2)

REPORTABLE OUTCOMES:

Manuscript in Preparation: Gmeiner, W.H., Boyacioglu, O., Jennings-Gee, J. "The Cytotoxic and Pro-Apoptotic Activities of the Novel Fluoropyrimidine F10 Towards Prostate Cancer Cells are Enhanced by Zn2+-Chelation and Inhibiting the Serine Protease Omi/HtrA2" In Preparation (appendix #1).

Manuscript in Preparation: Godwin, R. and Salsbury Jr., F. "NEMO Molecular Dynamics Simulations – effects of Zn2+ binding In Preparation (appendix #2).

Idea Award 2013 grant application: "Zn2+ Modulation for Treatment of Advanced Prostate Cancer" (Gmeiner PI)

Letter of intent for Synergistic Idea award mechanism "Omi/HtrA2 as a Novel Target for Prostate Cancer Treatment" (Gmeiner & Salsbury co-PIs)

CONCLUSION:

Prostate cancer cells tightly regulate Zn2+ levels and although this level is maintained at a much lower level in prostate cancer cells relative to normal prostate these cells are highly stressed by Zn2+ chelation and also by treatment with exogenous Zn2+. The combination of Zn2+ chelation and chemotherapy may be a highly effective strategy for prostate cancer treatment. The serine protease Omi/HtrA2 is newly idenitified as a Zn2+-responsive mediator of survival/paoptosis in prostate cancer cells. The combination of compuationald and experimental studies holds great promise for understanding the molecular interactions responsible for the response of prostate cancer cells to modulation of Zn2+ in combination with chemotherapy.

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APPENDIX:

Gmeiner, W.H., Boyacioglu, O., Jennings-Gee, J. "The Cytotoxic and Pro-Apoptotic Activities of the Novel Fluoropyrimidine F10 Towards Prostate Cancer Cells are Enhanced by Zn2+-Chelation and Inhibiting the Serine Protease Omi/HtrA2" In Preparation (appendix #1).

Godwin, R. and Salsbury Jr., F. "NEMO Molecular Dynamics Simulations – effects of Zn2+ binding In Preparation (appendix #2).

Appendix # 1

In Preparation December 2013

The Cytotoxic and Pro-Apoptotic Activities of the Novel Fluoropyrimidine F10 Towards Prostate Cancer Cells are Enhanced by Zn2+-Chelation and Inhibiting the Serine Protease Omi/HtrA2

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Grant sponsor: DOD CDMRP; NIH-NCI CA

BACKGROUND. Intracellular Zn2+ levels decrease during prostate cancer progression and agents that modulate intracellular Zn2+ are cytotoxic to prostate cancer cells by an incompletely described mechanism. F10 is a new polymeric fluoropyrimidine drug-candidate that displays strong activity with minimal systemic toxicity in pre-clinical models of prostate cancer and other malignancies. The effects of exogenous Zn2+ or Zn2+ chelation for enhancing F10 cytotoxicity are investigated as is the role of Omi/HtrA2, a serine protease that promotes apoptosis in response to cellular stress.

METHODS. To test the hypothesis that the pro-apoptotic effects of F10 could be enhanced by modulating intracellular Zn2+ we investigated cell-permeable and cell-impermeable Zn2+ chelators and exogenous Zn2+ and evaluated cell viability and apoptosis in cellular models of castration-resistant prostate cancer (CRPC; PC3, C4-2). The role of Omi/HtrA2 for modulating apoptosis was evaluated by pharmacological inhibition and Western blotting.

RESULTS. Exogenous Zn2+ initially reduced prostate cancer cell viability but these effects were transitory and were ineffective at enhancing F10 cytotoxicity. The cell-permeable Zn2+-chelator TPEN induced apoptosis in prostate cancer cells and enhanced the pro-apoptotic effects of F10. The pro-apoptotic effects of Zn2+-chelation in combination with F10 treatment were enhanced by inhibiting Omi/HtrA2 implicating this serine protease as a novel target for prostate cancer treatment.

CONCLUSIONS. Zn2+-chelation enhances the pro-apoptotic effects of F10 and may be useful for enhancing the effectiveness of F10 for treatment of advanced prostate cancer. The serine protease Omi/HtrA2 modulates Zn2+-dependent apoptosis in prostate cancer cells and represents a new target for treatment of CRPC.

KEY WORDS: **Zn2+, castration-resistant prostate cancer, F10, fluoropyrimidine, Omi/HtrA2**

INTRODUCTION. The relationship between Zn^{2+} and prostate cancer incidence, response to chemotherapy, and recurrence is complex. In general, prostate cancer cells have low intracellular Zn2+ levels (Costello and Franklin, 2011) although Zn2+ levels may vary during disease progression (Banas et al., 2010). Further, increased dietary Zn2+ is associated with prostate cancer survival (Epstein et al., 2011) although chronic zinc oversupply may enhance risk of prostate cancer (Leitzman et al., 2003). Either low or high dietary zinc increased tumor burden in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model of prostate cancer. Low levels of Zn2+ and Fe2+ correlated with disease recurrence in prostate cancer patients (Sarafanov et al., 2011). The importance of Zn2+ levels for prostate cancer progression has resulted in development of imaging modalities to sense Zn2+ levels as well as therapeutic approaches to modulate Zn2+ to enhance chemotherapy. The sensitivity of prostate cancer cells to both exogenous Zn2+ and Zn2+ chelation suggests Zn2+ levels are tightly regulated and not simply minimized in prostate cancer cells.

The apparently complex relationship between Zn2+ levels and prostate cancer incidence and recurrence has resulted in studies to modulate intracellular levels of Zn2+ for therapeutic benefit. Zn2+ is transported into cells by the SLC39 (Zip-family) members and is transported out of cells by SLC30 (ZnT) zinc transporters (Franz et al., 2013). Decreased expression of Zip-family members is characteristic of prostate cancer and as a consequence treatment of PCa cells with Zn2+ salts (e.g. $ZnSO_4$) may have minimal effect on intracellular Zn2+ necessitating Zn2+-delivery with a cell-permeable chelate, such as pyrithione (ZnPy). Several studies have, however, reported that Zn2+ salts can be cytotoxic to prostate cancer cells. For example, zinc acetate was cytotoxic to PC3, LNCaP, and DU145 cells and direct intratumoral injection decreased tumor growth. (Shah et al., 2009).

Other studies however report that while zinc salts are readily taken up and retained by myeloid progenitor cells and protect these cells from the pro-apoptotic effects of docetaxel chemotherapy similar treatment does not result in increased Zn2+ levels in prostate cancer cells or protect these cells from docetaxel-induced apoptosis (Makhov et al., 2011). In contrast to Zn2+ salts, treatment with ZnPy increased Zn2+ levels in prostate cancer cells and was cytotoxic to DU145, PC3, and LNCaP cells (Kriedt et al., 2010) and enhanced PTX- and TNFα-mediated apoptosis in PC3 cells (Uzzo et al., 2006). Thus, the effects of Zn2+ on prostate cancer cells depends on the cell permeability of administered Zn2+ as well as on the chemotherapy co-administered.

The present studies focus on modulating intracellular levels of Zn2+ in prostate cancer cells to enhance the effectiveness of chemotherapy with F10, a novel polymeric fluoropyrimidine that has shown promising activity in pre-clinical models of highly lethal malignancies including acute myeloid leukemia (AML) and glioblastoma (GBM). Our laboratory is interested in the potential of F10 for improved treatment of prostate cancer based upon results from the NCI 60 cell line screen indicating that cellular models of castration-resistant prostate cancer (CRPC; e.g. PC3, DU145) were highly sensitive to F10 with GI_{50} values in the nanomolar range. F10 displays large advantages relative to current chemotherapy and PC3 cells were more than 600-fold more sensitive to F10 relative to the conventional fluoropyrimidine drug 5-fluorouracil (5FU). These results suggest that F10 might be useful for treating CRPC despite clinical studies with 5FU and capecitabine demonstrating these drugs lack efficacy for prostate cancer treatment [1-3]. We report herein investigations of treatment effects in cellular models of CRPC (C4-2 and PC3) with exogenous Zn2+ as well as with Zn2+-chelators both as singe agents and in combination with F10. The cell

permeable Zn2+ chelator TPEN displays strong single agent activity and enhanced the pro-apoptotic effects of F10 towards PC3 and C4-2 cells demonstrating the potential for Zn2+-chelation to be used to enhance F10 efficacy for CRPC treatment.

The molecular targets affected by Zn2+-treatment in prostate cancer and other cells have been partly elucidated and include mediators of apoptosis such as caspase 3 and the caspase 3 substrate X-linked inhibitor of apoptosis (XIAP). Zn2+ inhibits the activity of caspase 3 (Perry et al., 1997) and cell-permeable Zn2+ chelators including TPEN (Chimienti et al., 2001) and PAC-1 (Peterson et al., 2009) induce apoptosis through increased caspase 3 activation. Zinc depletion also results in cleavage of XIAP prior to induction of apoptosis in prostate cancer cells in a caspase-independent as well as proteasome-independent manner [4]. The Zn2+-dependent cleavage of XIAP is selective and neither cIAP1 nor cIAP2 are similarly affected by Zn2+ chelation. Zn2+-dependent XIAP cleavage is inhibited by Pefabloc implicating a serine protease in mediating these effects. A potential candidate Ser protease for Zn2+-dependent XIAP degradation is Omi/HtrA2. Omi/HtrA2 undergoes auto-catalysis to release a form that has homology to Smac/DIABLO[5]. This cleaved product has pro-apoptotic properties resulting from sequestering XIAP, and thus indirectly activates caspase 3[6]. Omi/HtrA2 is located in the mitochondria, however upon pro-apoptotic stimulation processed Omi/HtrA2 translocates into the cytosol and binds XIAP. Although Omi/HtrA2 is known to be important for promoting apoptosis in response to cellular stress[7], it has not been previously reported to contribute to apoptosis in PCa cells. We report that Omi/HtrA2 inhibition has single agent activity and induces apoptosis in prostate cancer cells. Our studies also show that inhibiting Omi/HtrA2 enhances F10-induced apoptosis (and synergizes) with F10 towards CRPC cells. These studies demonstrate Omi/HtrA2 is a novel target for prostate cancer treatment.

Materials and Method

Cell Maintenance: C4-2 cell line was a gift from Dr. Elizabeth M. Wilson (UNC, Chapel Hill, NC). PC3 cells were purchased from cell and viral vector core laboratory at Wake Forest School of Medicine. All cells were maintained with RPMI 1640 (Gibco, Grand island, NY) with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA). All cells were kept at 5% CO_2 at 37 °C.

Zn2+ and Drug Treatment. F10 was synthesized as previously described. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless indicated otherwise.

Zinc cytotoxicity in the form of ZnP, NaP, and $ZnSO_4$: PC3 and C4-2 cells were seeded at a density of 3,000 cells/well in 96-well plates and incubated at 37 °C under 5% CO_2. Next day, the cells were treated with ZnP, NaP, or ZnSO4 for 72 h. Live cell counts were measured indirectly by measuring the ATP amounts using CellTiter-Glo® luminescent cell viability assay (Promega, Madison, WI) according to the manufacturer's protocol.

Co-treatment with FdUMP[10] and ZnP: PC3 and C4-2 cells were seeded at a density of 3,000 cells/well in 96-well plates and incubated at 37 °C under 5% CO_2. Next day, the cells were treated with FdUMP and ZnP for 48-72 h. Total ATP levels were measured at the end of the treatment time using CellTiter-Glo® luminescent cell viability assay.

Intracellular Zinc Measurements: PC3 and C4-2 cells were seeded at a density of 3,000 cells/well in 96-well plates and incubated at 37 °C under 5% CO_2. Four days later, the media was removed and cells were incubated under minimal light for 30 min at 37 °C with 1

μM of FluoZin™-3 AM (Molecular Probes, Eugene, OR) in phosphate-free, Hepes-buffered Hanks' balanced salt solution (HHBSS) (KCl 0.4 g, $NaHCO_3$ 0.35 g, $CaCl_2$ 0.14 g, $MgCl_2 \cdot 6H_2O$ 0.1 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, NaCl 8 g, D-glucose 1 g, Hepes 5.2 g for 1 liter volume) since phosphate precipitates Zn^{2+} in solution. After 30 min, the buffer with FluoZin-3 AM was discarded and the cells were incubated with ZnP in HHBSS for 1 h at 37 °C followed by twice wash with HHBSS. Fluorescence emitted by the Fluozin-3 AM that bound Zn2+ and trapped inside the cells was measured at using (the plate reader in Debinski Lab) excitation/emission wavelengths of 485/520 nm.

Microscopy

Western blots

Statistical Analysis

RESULTS

Cytotoxicity of Exogenous Zn2+ and Zn2+-Chelation. The effects of exogenous Zn2+ or of Zn2+ chelation on the viability of prostate cancer cells were investigated using PC3 and

C4-2 cells treated with either zinc sulfate or with the cell permeable zinc chelator pyrithione as either the sodium or zinc complex. Alternatively, prostate cancer cells were treated with the cell impermeable Zn2+ chelator DTPA to decrease Zn2+ concentrations in culture media and indirectly decrease intracellular Zn2+. The results revealed a marked time-dependence to the effects of both exogenous Zn2+ and its chelation on prostate cancer cell viability (**Figure 1**). Treatment with zinc sulfate initially decreased cell viability, particularly for C4-2 cells, however 72 h of continuous treatment with elevated exogenous Zn2+ resulted in no significant change to the viability of PC3 cells and only a moderate (~20%) decrease in the viability of C4-2 cells consistent with cellular adaptation to increased Zn2+ in culture medium. The effects of zinc salts on cell viability were independent of the counterion with zinc chloride as effective as zinc sulfate demonstrating the specificity of cell viability effects on Zn2+ levels.

Pyrithione is a pyridine N-oxide that forms a 2:1 complex with Zn2+ that is cell permeable and widely used for studies evaluating the effects of increased intracellular Zn2+ on cell function independent of cell transport. Our studies show that sodium pyrithione that cannot deliver exogenous Zn2+ is cytotoxic to both C4-2 and PC3 cells in a time-dependent manner consistent with pyrithione affecting cell viability independent of Zn2+ delivery. Pyrithione cytotoxicity is however enhanced when combined with exogenous Zn2+ - conditions that enhance cell-uptake of Zn2+ independent of Zn2+ transporter function (**Figure 1**). The combination of pyrithione and Zn2+ shows reduced effects on cell viability at later timepoints as was observed for Zn2+-only treatment consistent with prostate cancer cells adapting to increased intracellular Zn2+ at timepoints longer than 24 h. The results demonstrate that pyrithione affects prostate cancer viability independent of Zn2+ transport while the zinc complex exerts Zn2+ specific effects on cell viability.

While exogenous Zn2+ decreases the viability of prostate cancer cells, Zn2+ chelation with the cell impermeable Zn2+ chelator DTPA also decreases viability consistent with cellular homeostasis requiring tight regulation of intracellular Zn2+ that is dependent on the concentration of free Zn2+ in culture medium. The reduction in prostate cancer cell viability due to chelating Zn2+ in culture medium is slower than effects resulting from exogenous Zn2+ treatment with significant reduction in viability at 72 h but not at 24 h of treatment (**Figure 1**). The cytotoxic effects of DTPA-treatment were completely reversed by co-treatment with zinc sulfate consistent with DTPA cytotoxicity resulting from Zn2+ chelation. Sodium pyrithione enhanced the effects of DTPA and this enhancement was reversed by zinc sulfate consistent with pyrithione cytotoxicity being Zn2+-dependent.

Cell Imaging of Intracellular Zn2+. The cytotoxic effects of exogenous Zn2+ were investigated using the cell-permeable Zn2+-sensitive fluorescent dyes Zinquin and FluoZin-3 (**Figure 2**). Treatment of both PC3 and C4-2 cells with ZnPy resulted in concentration-dependent increases in FluoZin fluorescence in cells pre-treated with FluoZin consistent with intracellular transport of Zn2+ being responsible for the cytotoxicity of ZnPy. The sub-cellular distribution of fluorescence resulting from FluoZin and Zinquin treatment was distinct although both fluorophores displayed strong concentration-dependent fluorescence that was responsive to ZnPy treatment consistent with the observed fluorescence being correlated with Zn2+ uptake into prostate cancer cells. Zinquin fluorescence was punctate consistent with sequestration into endosomes, lysososmes or other organelles while FluoZin fluorescence was diffuse consistent with predominantly cytoplasmic distribution. Intriguingly, treatment with the cell-permeable Zn2+ chelator TPEN, which is also cytotoxic to prostate cancer cells, also caused a much more modest yet reproducible increase in

fluorescence monitored by either Zinquin and FluoZin pre-treatment consistent with perturbation of Zn2+ levels. While the origin of Zn2+-dependent fluorescence by Zinquin and FluoZin remains under investigation and apparently involves interaction of the fluorphores with Zn2+-binding proteins rather than with an aqueous free-Zn2+ pool in cells, the results are consistent with ZnPy cytotoxicity being mediated through increased intra-cellular Zn2+ levels.

ZnPy Combination Effects with Chemotherapy. Our studies demonstrated ZnPy decreased cell viability via increased intracellular Zn2+. To determine to what extent ZnPy treatment could be used in combination with chemotherapy we investigated co-treatment of ZnPy with F10, a novel polymer of 5-fluoro-2'-deoxyuridine-5'-O-monophosphate, the active metabolite of fluoropyrimidine chemotherapy that displays strong anti-cancer activity and minimal systemic toxicity in pre-clinical models of AML and GBM and that in previous studies demonstrated strong cytotoxicity towards prostate cancer cells. F10 reduced the viability of both PC3 and C4-2 cells with IC50 values in the nanomolar range (**Figure 3**). PC3 cells were markedly more sensitive to the effects of ZnPy relative to C4-2 cells although the IC50 for ZnPy in both cell lines exceeded 100 micromolar. Further, at concentrations of ZnPy that induced at least 50% reduction in viability for either cell line the effects of ZnPy treatment were independent of F10 concentration consistent with higher concentrations of ZnPy interfering in F10 cytotoxicity. Interestingly, while either F10 or ZnPy reduced the viability of prostate cancer cells only F10 induced apoptosis and ZnPy at higher concentrations inhibited F10-induced apoptosis. ZnPy did decrease levels of full-length Caspase 3 however the cleaved fragment p16 was not detected and caspase 3/7 glo assays did not indicate the occurrence of end-stage apoptosis. Both ZnPy and F10 resulted in decreased levels of pAkt consistent with induction of changes in cellular signaling that

disfavor cell proliferation. As F10 cytotoxicity is highly dependent on cell proliferation, the decreased levels of pAkt that occur upon ZnPy treatment may reduce DNA replication and cell division providing the basis for minimizing the cytotoxic and apoptotic effects of F10.

Intracellular Zinc Chelation is Cytotoxic and Enhances F10-induced Apoptosis: In contrast to DTPA that is cell impermeable, the Zn2+ chelator TPEN is readily internalized by prostate cancer cells (**Figure 4**). Our studies demonstrated that treatment with TPEN reduced the viability of prostate cancer cells and that the effects of TPEN were more rapid than occurred via chelation of Zn2+ in culture medium with DTPA. TPEN treatment also significantly reduced FluoZin fluorescence in C4-2 cells, consistent with effects on cell viability being Zn2+-mediated. Further, the effects of TPEN treatment on cell viability were completely reversed by co-treatment with zinc sulfate as was reduction in FluoZin fluorescence. Co-treatment of PC3 and C4-2 cells with TPEN during the final six hours of F10 treatment resulted in significant reduction in cell viability and increased apoptosis. The combination of F10 and TPEN was synergistic (Chris – can you check this and get a CI value?) and the effects of TPEN were negated by co-treatment with Zn2+. Treatment with F10 and F10 + TPEN resulted in increased expression of p53-responsive pro-apoptotic genes p21 and Bax in C4-2 cells consistent with F10 inducing apoptotic death and Zn2+-chelation with TPEN stimulating execution of apoptosis.

We then sought to determine if the pro-apoptotic effects of Zn2+-chelation might be mediated by the serine protease Omi/HtrA2. Omi/HtrA2 undergoes autocatalysis to release a product that has homology to Smac/DIABLO and which has pro-apoptotic properties resulting from sequestering the anti-apoptotic protein XIAP. As the apoptotic effects of Zn2+-chelation are mediated via caspase 3 activation which in turn is inhibited by XIAP

Omi/HtrA2 activation may be expected to enhance apoptosis by sequestering XIAP. Changes in Omi/HtrA2 expression were evaluated by Western blot and the effects of Omi/HtrA2 inhibition for mitigating the apoptosis-stimulating effects of Zn2+-chelation in F10-treated cells were evaluated using the inhibitor UCF-101. Western blot revealed no change in the expression of Omi/HtrA2 under any conditions examined. Surprisingly, however, inhibiting Omi/HtrA2 enhanced the cytotoxic and pro-apoptotic effects of F10 and further enhanced the pro-apoptotic effects of Zn2+-chelation. These results reveal an anti-apoptotic role for Omi/HtrA2 in F10-treated cells and identify Omi/HtrA2 as a putative therapeutic target for treatment of prostate cancer.

DISCUSSION

Castration-resistant prostate cancer remains incurable with chemotherapy offering only a moderate survival advantage that is achieved with treatment regimens that compromise patient quality of life. Pre-clinical studies with the fluoropyrimidine polymer F10 have demonstrated this novel drug-candidate displays excellent anti-cancer activity in pre-clinical animal models of acute myeloid leukemia (AML) and glioblastoma, two highly lethal malignancies for which new treatment options are also urgently needed. Data from the NCI 60 cell line screen as well as from our laboratory have demonstrated that F10 is highly cytotoxic to prostate cancer cells with the present studies demonstrating F10 is cytotoxic to C4-2 and PC3 cells at nanomolar levels. He present studies have demonstrated that the cytotoxic effects of F10 towards prostate cancer cells can be enhanced by modulating

intracellular Zn2+ levels providing the basis for developing new therapeutic modalities that simultaneously deliver F10 and modulate Zn2+ for therapeutic benefit.

The progression of prostate cancer is paralleled by sequentially decreased levels of intracellular Zn2+ consistent with low levels of Zn2+ in prostate cancer cells presenting a survival advantage. Consistent with this interpretation prostate cancer cells in culture as well as in human malignant tissue express decreased levels of zinc transporters and increased levels of Zn2+ efflux pumps. Further, administering exogenous Zn2+ has been found to decrease prostate cancer cell viability and studies from our laboratory demonstrate that strategies to deliver Zn2+ enhance the cytotoxicity of chemotherapy under some conditions. The present studies demonstrate that treatment of prostate cancer cells with Zn2+ salts initially decreases cell viability, however these effects are transitory and cell recover full viability within 72 h. The cell permeable ionophore pyrithione reduces prostate cancer cell viability as the sodium complex and these effects are enhanced for the Zn2+ complex (Zn-Py). As with cell impermeable Zn2+-salts, the effects of Zn2+-Py are decreased at longer treatment times consistent with cellular adaptation to increased Zn2+. Further, Zn2+-Py is effective at enhancing the cytotoxicity of F10 only at low concentrations with higher concentration of Zn-Py that have substantial single-agent effects inhibiting F10 cytotoxicity. The finding that Zn-Py reduces pAkt levels is consistent with Zn-Py promoting cytotostasis thus limiting the effectiveness of F10 which is proliferation dependent.

Although prostate cancer cells maintain Zn2+ levels at very low levels relative to normal prostate, further reduction in intracellular Zn2+ through Zn2+ chelation results in apoptosis. These observations are consistent with low levels of Zn2+ being important for maintaining a pro-survival balance in prostate cancer cells. Our studies show that both cell-impermeable

(e.g. DTPA) and cell-permeable (e.g. TPEN) Zn2+-chelators each reduce the viability of prostate cancer cells. The effects of DTPA are considerably slower than TPEN demonstrating that cell-permeable Zn2+ chelation has potential for rapid induction of apoptosis in prostate cancer cells. Further, the combination of F10 and TPEN is synergistic (CI = ?) demonstrating the potential for cell-permeable Zn2+ chelation to be used to enhance the efficacy of F10 chemotherapy. The mechanism by which Zn2+-chelation enhances F10-induced apoptosis is not known, however Zn2+ has been shown to form a specific complex with Cysxxx in caspase 3 that inhibits apoptosis. F10-induced apoptosis may be attenuated though Zn2+-complexation of caspase 3 with Zn2+-chelation overcoming this attenuation.

The mechanism by which Zn2+-chelation tips the survival/apoptosis balance to apoptosis in prostate cancer cells is not known. Previous studies demonstrated TEPN treatment resulted in selective depletion of the X-linked inhibitor of apoptosis (XIAP) and this degradation was prevented by the serine protease inhibitor Pefabloc. The present studies investigated a potential role for the serine protease Omi/HtrA2 in the Zn2+-dependent degradation of XIAP. Omi/HtrA2 undergoes auto-catalysis to release a form that has homology to Smac/DIABLO and that has pro-apoptotic properties resulting from sequestering XIAP. Omi/HtrA2 has also been shown to promote apoptosis in response to cellular stress. Surprisingly, our results implicate an anti-apoptotic role for Omi/HtrA2 with pharmacological inhibition markedly enhancing F10- and F10/TPEN-induced apoptosis. These results identify Omi/HtrA2 as a novel target for prostate cancer treatment with inhibitors of Omi/HtrA2 may have single agent activity as well as enhance the pro-apoptotic activities of chemotherapy towards prostate cancer cells.

CONCLUSION

Zn2+-chelation with cell permeable moieties represents a promising strategy for prostate cancer treatment that may also enhance the efficacy of chemotherapy. TEPN treatment enhances F10-induced apoptosis in PC3 and C4-2 cells demonstrating the potential for this combination therapy approach to be further evaluated in pre-clinical models of castration-resistant prostate cancer. In light of the low toxicity and strong anti-cancer activity of F10 in animal models of AML and GBM this combination is likely to be effective and well-tolerated for prostate cancer treatment. The serine protease Omi/HtrA2 contributes to prostate cancer cell survival and pharmacological inhibition of Omi/HtrA2 enhances the pro-apoptotic properties of F10 and F10/TPEN. These studies identify Omi/HtrA2 as a potential new target for prostate cancer treatment.

ACKOWLEDGEMENT

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protease Omi/HtrA2 regulates apoptosis by binding XIAP through a reaper-like motif. J Biol Chem, 2002. **277**(1): p. 439-44.
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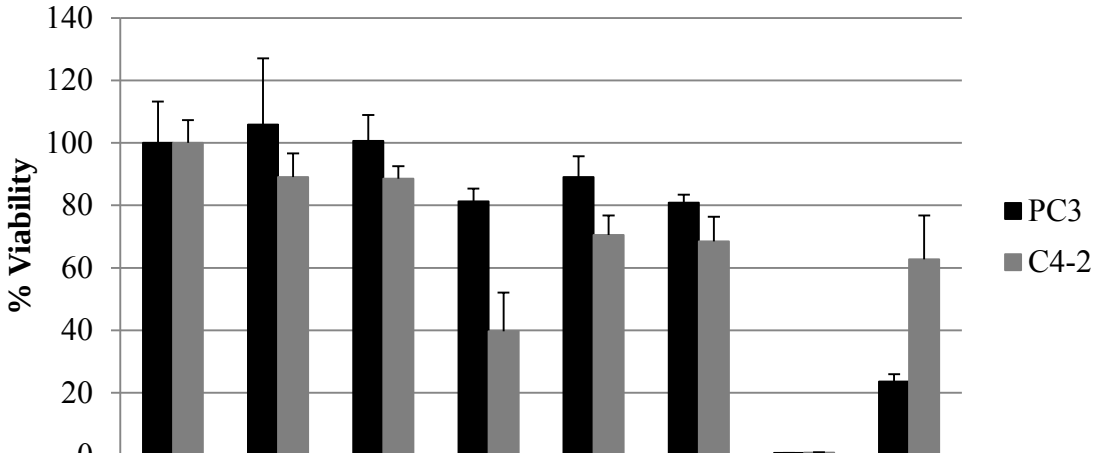
Supporting Information.

Draft Figures

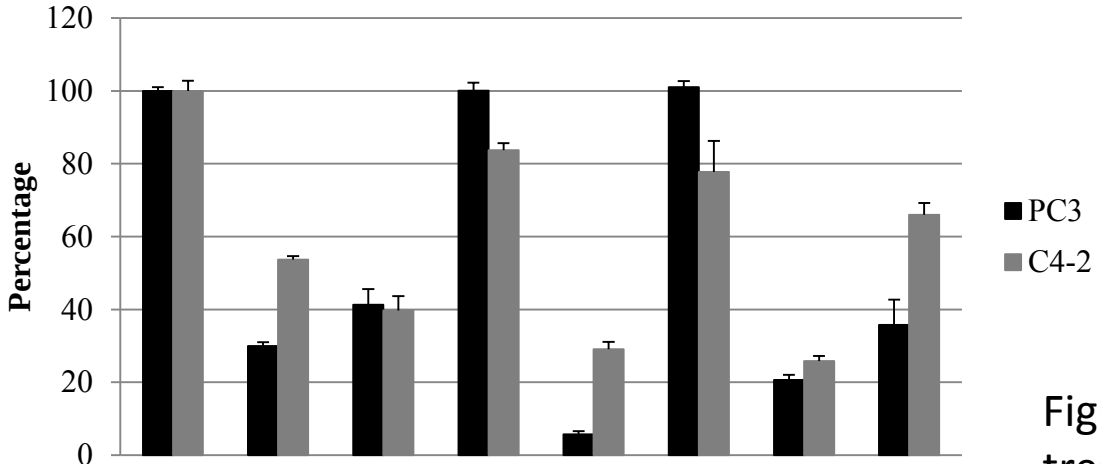
The Cytotoxic and Pro-Apoptotic Activities of the Novel Fluoropyrimidine F10 Towards Prostate Cancer Cells are Enhanced by Zn2+-Chelation and Inhibiting the Serine Protease Omi/HtrA2

William H. Gmeiner, Olcay Boyacioglu, Jamie Jennings-Gee

24 h



72 h



50 µM DTPA		+			+	+		+
1.2 µM NaP			+		+		+	+
50 µM $ZnSO_4$				+		+	+	+

Figure 1: Effect of treatment with the cell-impermeable Zn2+ chelator DTPA or cell permeable sodium pyrithione w/wo Zn2+ for the indicated times in PC3 and C4-2 cells.

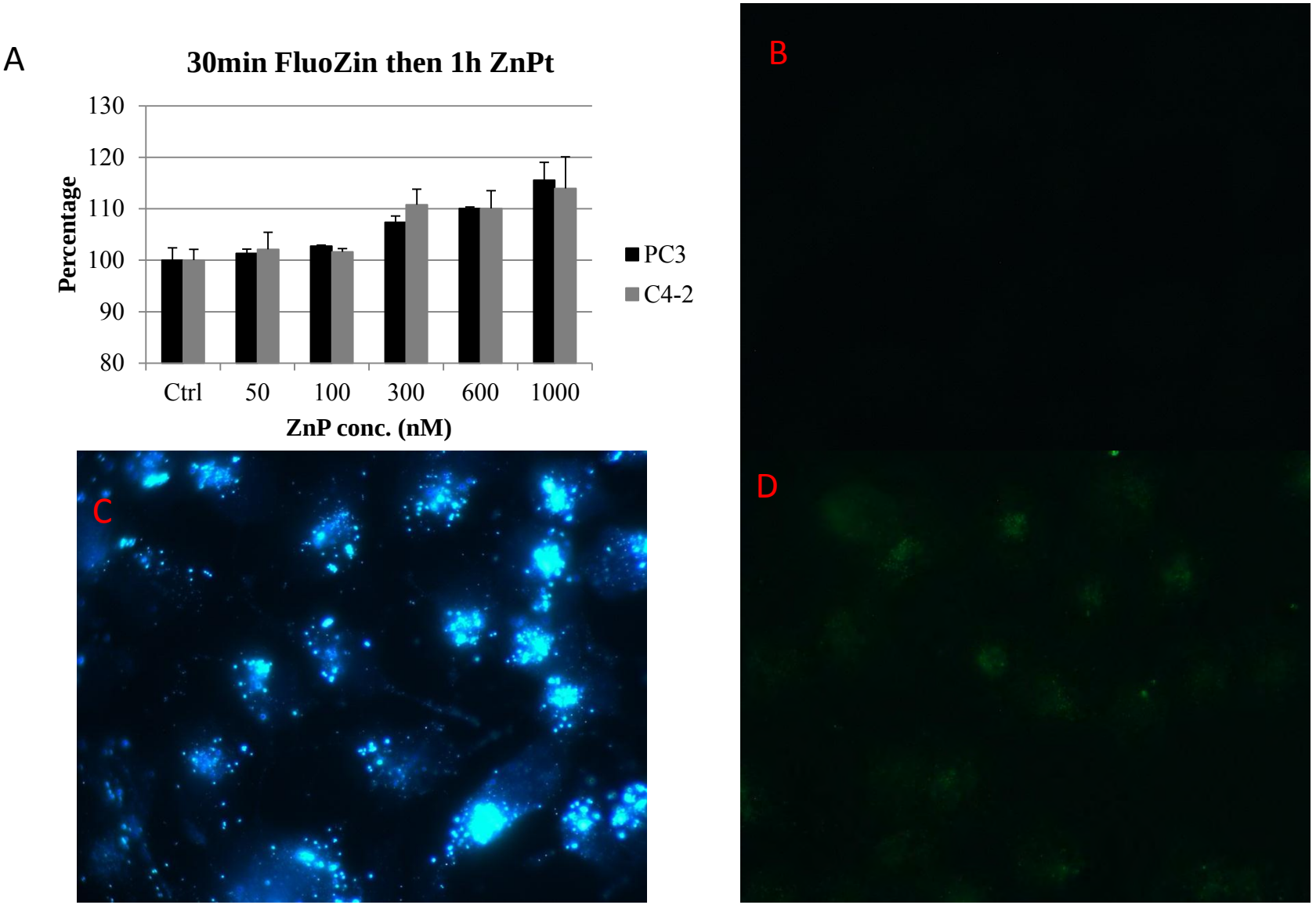
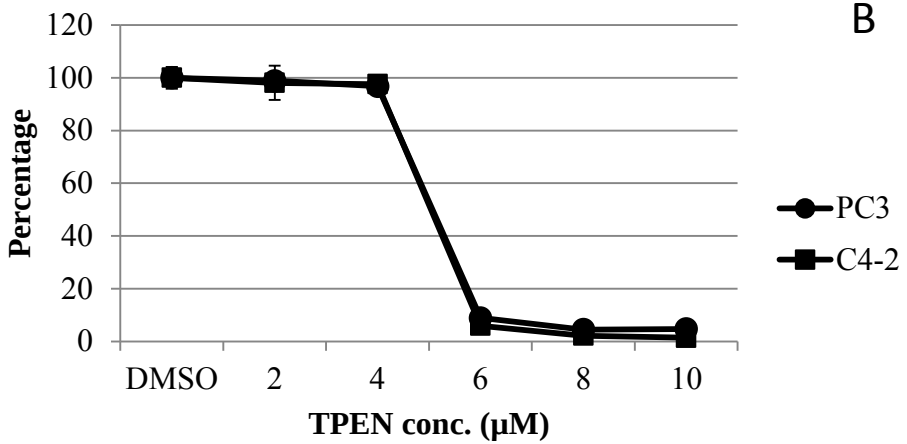
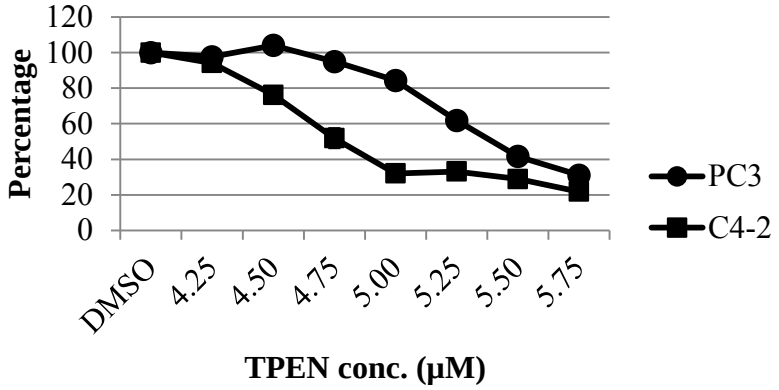


Figure 2 – Change in FluoZin fluorescence following treatment with Zn2+ pyrithione at the indicated concentrations. B – D confocal images of C4-2 cells following treatment with B – no treatment; C – 6 microM ZnPt Zinquin detection; D – 6 microM ZnPt FluoZin detection

A

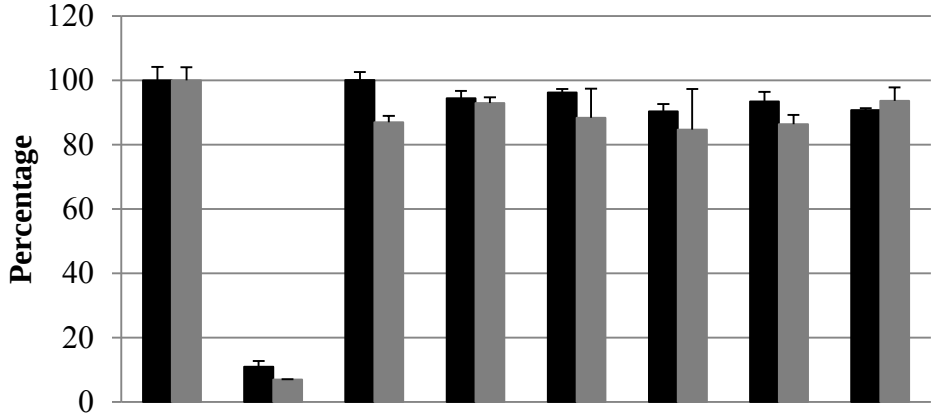


B



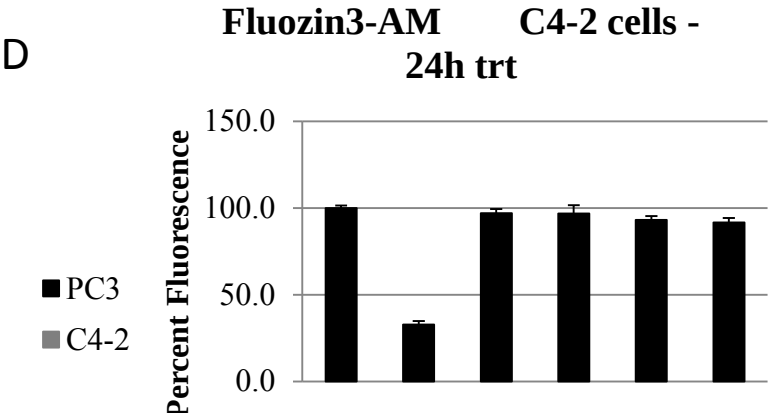
TPEN 24 h treatment

C



6 µM TPEN		+	+	+	+	+	+	+
2 µM $ZnSO_4$			after 0h		after 1h		after 2h	
6 µM $ZnSO_4$				after 0h		after 1h		after 2h

D



6 µM TPEN		+	+			
$ZnSO_4$			6 µM	2 µM	6 µM	10 µM

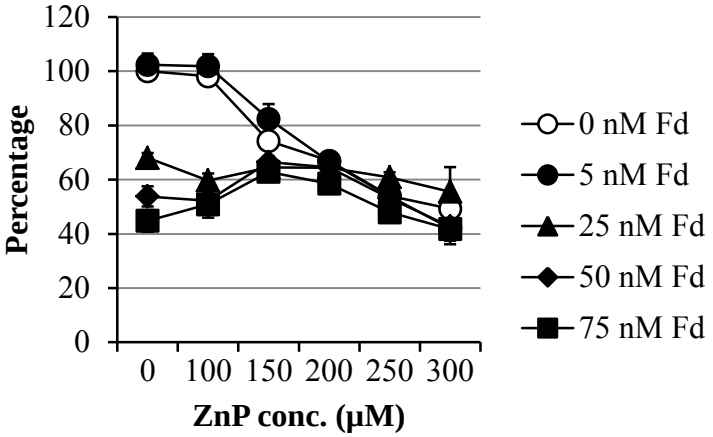
Figure 3 A,B effect of Zn2+ chelation with TPEN on PCa viability; C,D rescue of TPEN effects with Zn2+

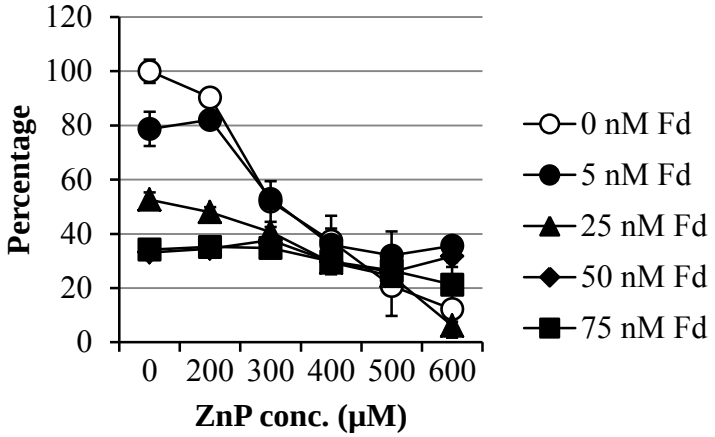
Figure 4. Effects of combining TPEN treatment with F10 chemotherapy on Pca viability and apoptosis

PC3 (48h)

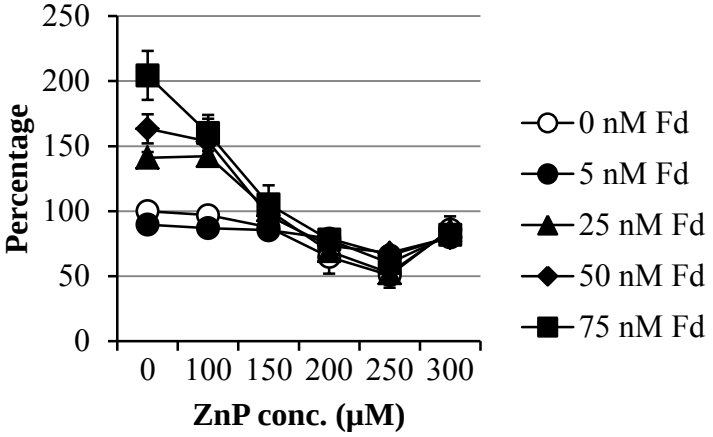
C4-2 (72 h)

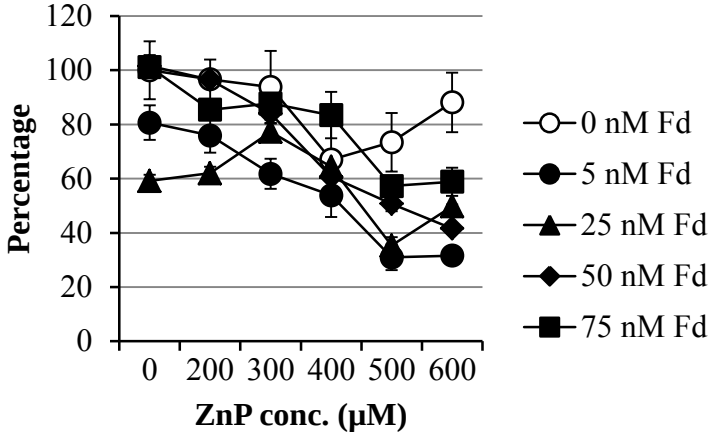
Viability





Casp 3/7





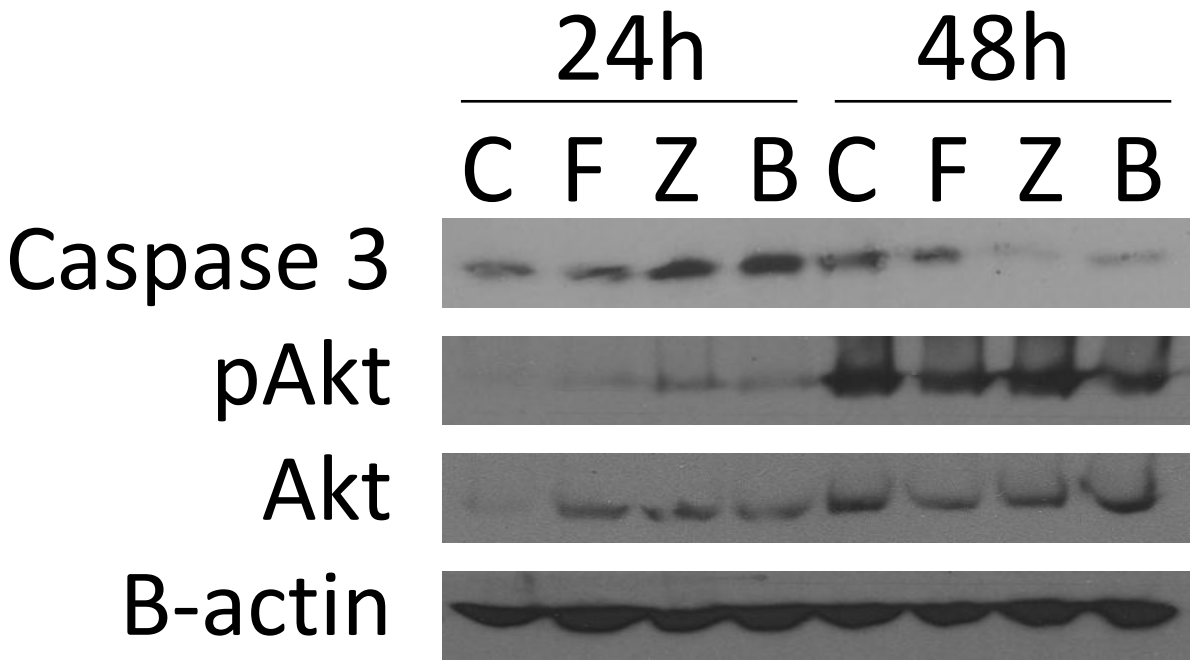
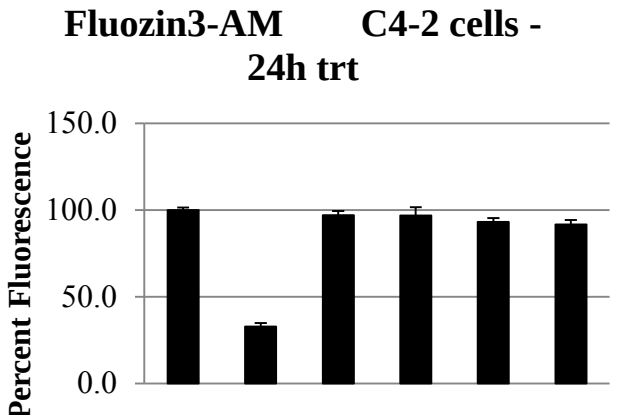


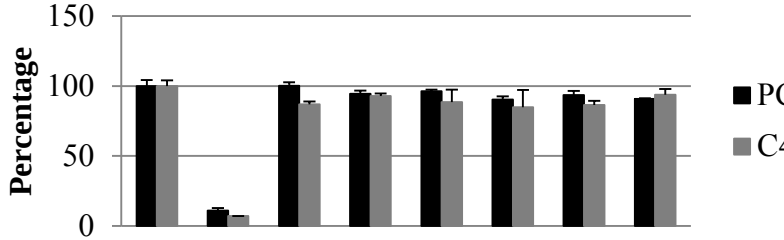
Figure 5. Western blot showing effects of F10 (F) or ZnPy (Z) or both treatments on levels of caspase 3, phosphorylation of Akt, and total Akt.

A



6 µM TPEN		+	+			
$ZnSO_4$			6 µM	2 µM	6 µM	10 µM

B



6 µM TPEN		+	+	+	+	+	+	+
2 µM $ZnSO_4$			after 0h		after 1h		after 2h	
6 µM $ZnSO_4$				after 0h		after 1h		after 2h

C

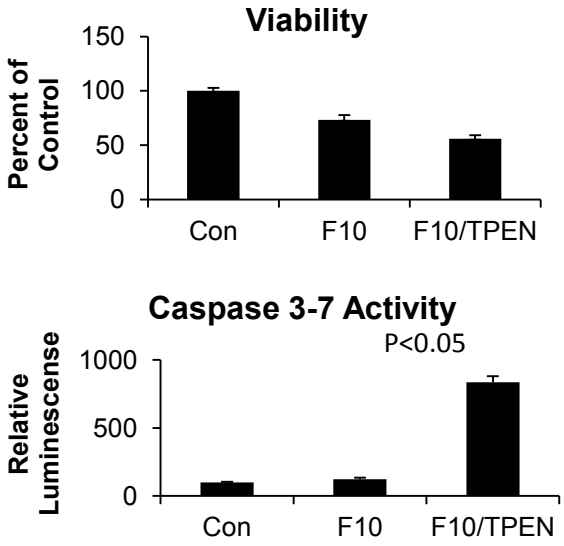


Figure 6. Effects of treatment with TPEN +/- Zn2+ on A) FLuoZin fluorescence; B Cell viability; C – effects of TPEN during last six hours of 48 h F10 treatment on C4-2 cell viability and apoptosis

Appendix #2

NEMO- All Atom Molecular Dynamics

Ryan Godwin
December 12, 2013

Submitted APS Abstract (11.15.13)

Conformational Analysis on structural perturbations of the zinc finger NEMO

Ryan Godwin & Freddie Salsbury

The NEMO (NF_κB Essential Modulator) Zinc Finger protein (2jvx) is a functional Ubiquitin-binding domain, and plays a role in signaling pathways for immune/inflammatory responses, apoptosis, and oncogenesis [*Cordier et al., 2008*]. Characterized by 3 cysteines and 1 histidine residue at the active site, the biologically occurring, bound zinc configuration is a stable structural motif. Perturbations of the zinc binding residues suggest conformational changes in the 423 atom protein characterized via analysis of all-atom molecular dynamics simulations. Structural perturbations include simulations with and without a zinc ion and with and without de-protonated cysteines, resulting in four distinct configurations. Simulations of various time scales show consistent results, yet the longest, GPU driven, microsecond runs show more drastic structural and dynamic fluctuations when compared to shorter duration time-scales. The last cysteine residue (26 of 28) and the helix on which it resides exhibit a secondary, locally unfolded conformation in addition to its normal bound conformation. Combined analytics elucidate how the presence of zinc and/or protonated cysteines impacts the dynamics and energetic fluctuations of NEMO.

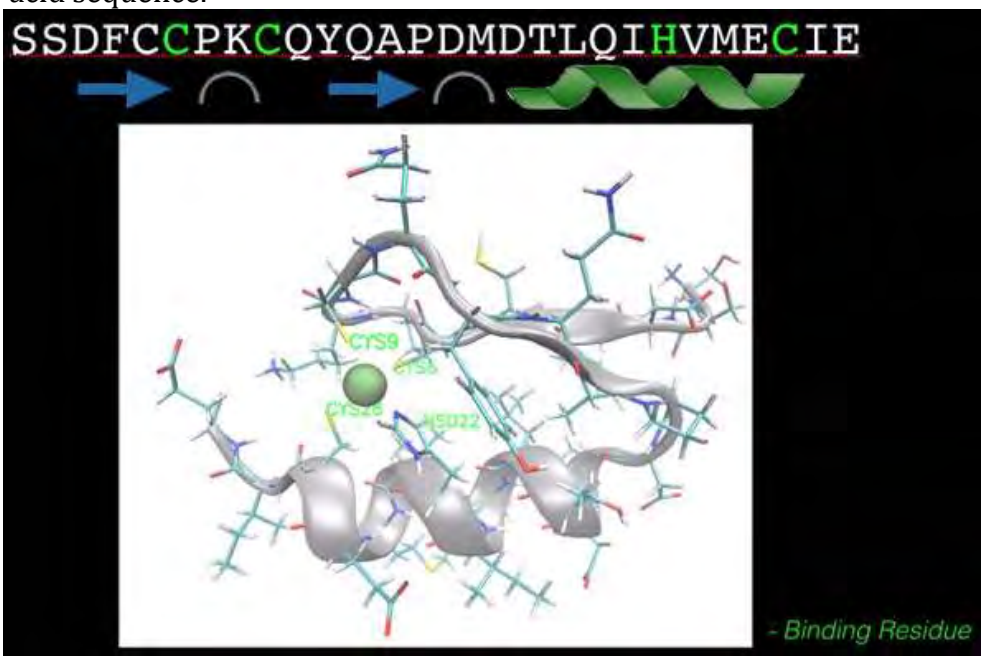
Summary to Date

There are two main ideas to express, and this document will (try to) focus on the later. The first idea is that nano-scale simulations are insufficient when examining energetics of proteins in these sorts of all-atom MD simulations. Microseconds and longer are really needed to provide enough sampling to understand kinetic changes available to the system. The second idea is the primary results of the simulations to date and corresponding analysis of the 4 configurations of the NEMO, with the microsecond simulations the focus of the analysis. Additional investigations of zinc finger proteins are underway (3CLX simulations are almost complete, 1I3O, 1C9Q & 2QRA are on deck) and we're investigating the incorporation of TPEN molecule into zinc finger simulations.

Background Material

NEMO is a 28 residue, 432 atom protein that binds a zinc ion at the 6^{th}, 9^{th}, 22^{nd}, and 26^{th} residues. These binding residues are cysteine, cysteine, histidine, cysteine,

respectively as highlighted in the image below. There are 2 beta sheets and an alpha helix along with two hairpin turns each approximated by the cartoon below the amino acid sequence.



A depiction of the protein including the secondary structures, residues and bound zinc (green orb) is also shown in the figure.

We investigated four configurations of the protein, as described in the abstract and highlighted in the following table. The thermodynamic cycle, including the two physiologically pertinent configurations, in bold, is:

With Zinc CYN	Without Zinc CYN
With Zinc CYS	**Without Zinc CYS**

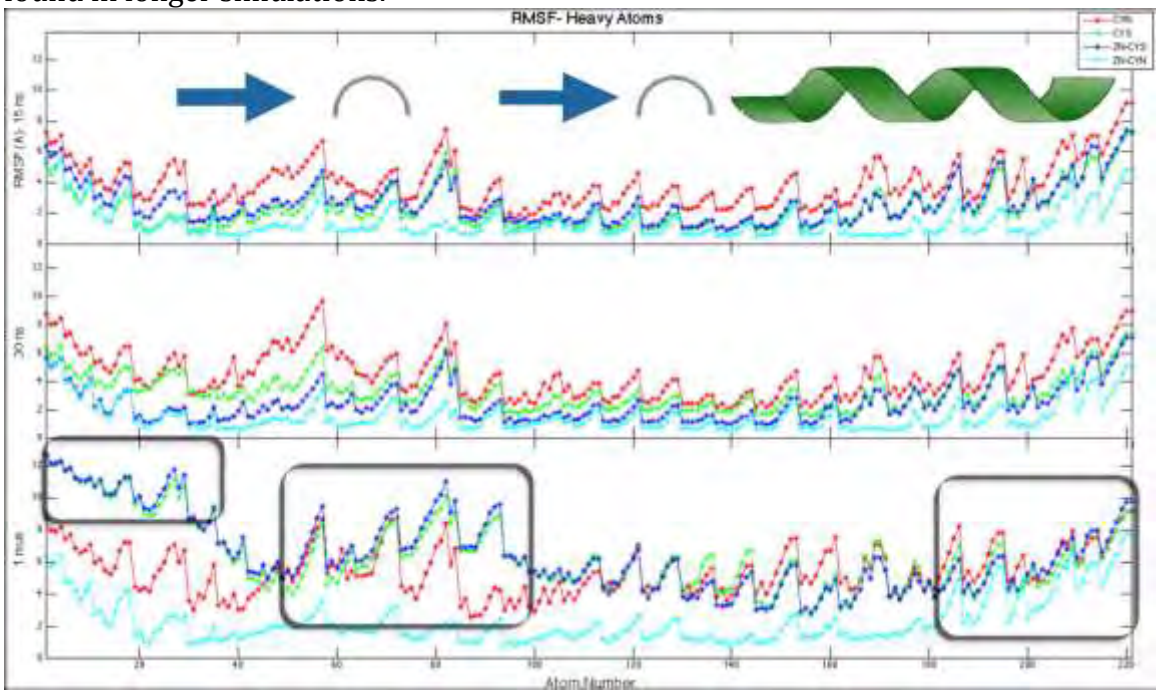
Simulations were recorded of various durations, and some sampling was gathered for the shorter scale simulations as highlighted in the following table

15 nanoseconds (300 ns sampling)
30 nanoseconds (300 ns sampling)
1 microsecond (1 mus sampling)

So let's move on to the more interesting stuff… data.

RMSF

RMSF values for all four configurations at each of the three time scales are all plotted below. The plots, from top to bottom are 15 ns, 30 ns, and 1 mus. The black squares highlight some of the larger effects, especially at the termini of the protein, found in longer simulations.



Clustering

Clustering seems to be as much of an art at the moment as a science. A cutoff of 4 angstroms of heavy atoms and 50 bins were used for the clustering parameters clustering 10000 frames of the microsecond trajectory. This was the best 'middle ground' distance to help cluster the more difficult to cluster non-biologically relevant states without sacrificing too much at the nicely behaved, biologically relevant regime.

Clusters

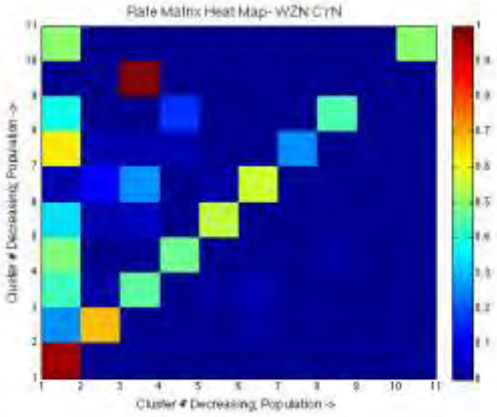
Clustering analysis is used to compare relative populations of states across the four structural configurations.

See supplemental Pdf

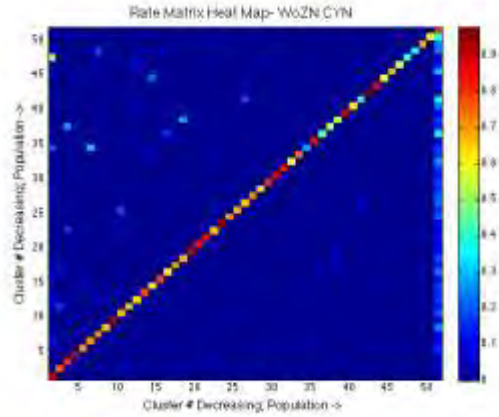
Markov Rate Matrix

The Markov Rate matrix shows the probability from transitioning from one state (along the y-axis) to another (along the x-axis). These types of plots can highlight kinetic traps, a particular state that has a very low probability of leaving that once it is found. It also highlights kinetically isolated states, which might interact with only one or two other states.

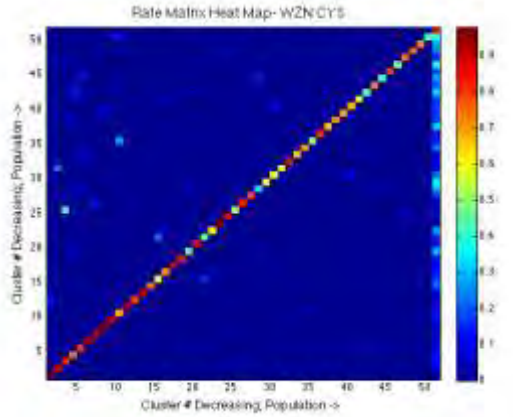
The converging probabilities checks that the states are expected to be found within a reasonable number of steps given that they start populated and unpopulated. The x-axis represents the number of steps and the y-axis is the ratio of the probabilities of being in that state to the final state.
First looking at WZN CYN there are only 9 states given the structural stability and the large cutoff distance.



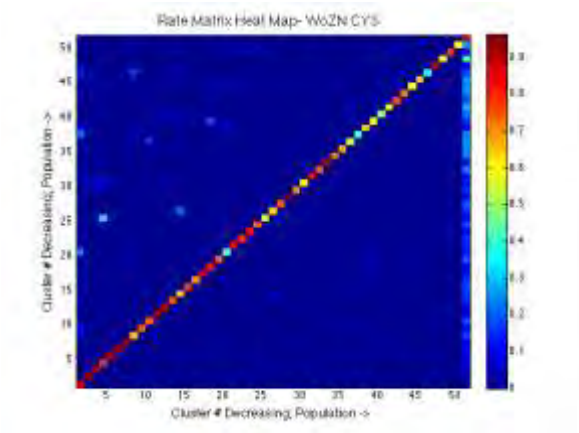
Now removing the zinc we'll look at the WoZN CYN case. There is definitely a higher order state (maybe 42) that is a kinetic trap. There is also a good deal of kinetic isolation in the middle states.



For the WZN CYS case, we have, again that the unclustered structures dominate and the low-lying states are rather kinetically isolated.



Finally, the other biologically relevant configuration has the following Markov chain.



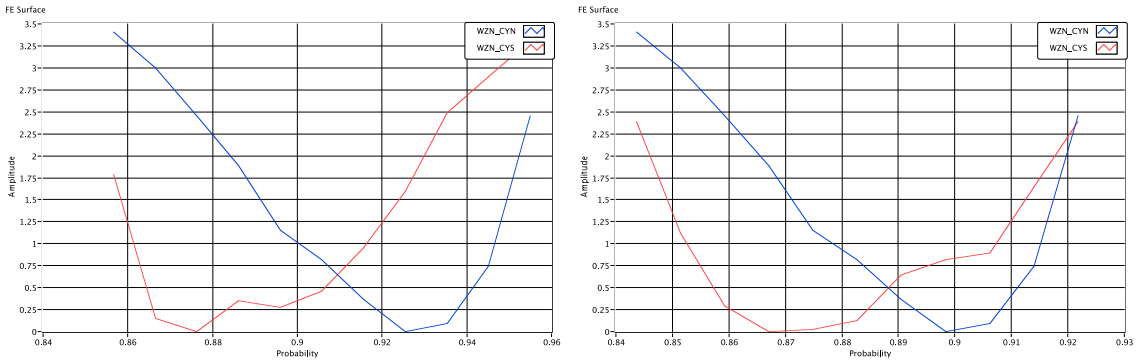
We see that all of the probabilities converge within 50 steps, supporting the Markov plots.

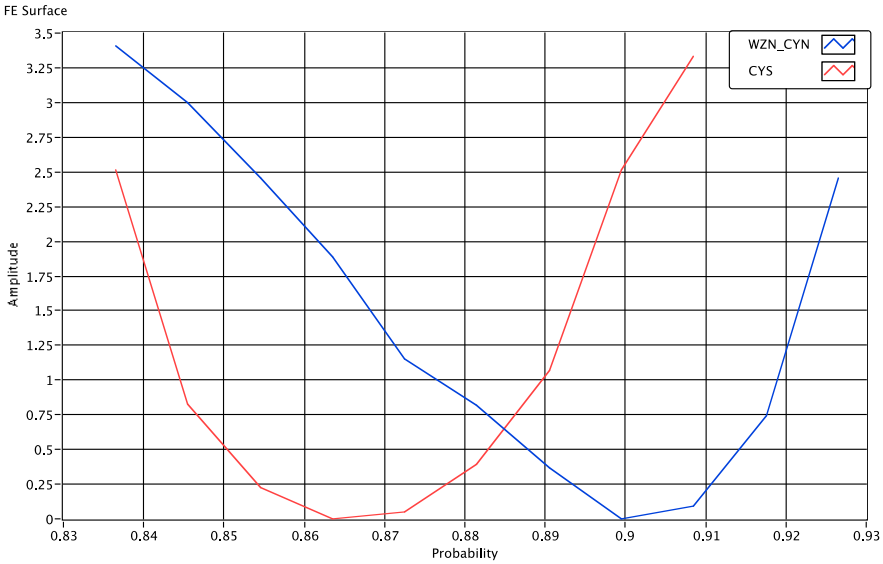
Correlation

Native Contacts

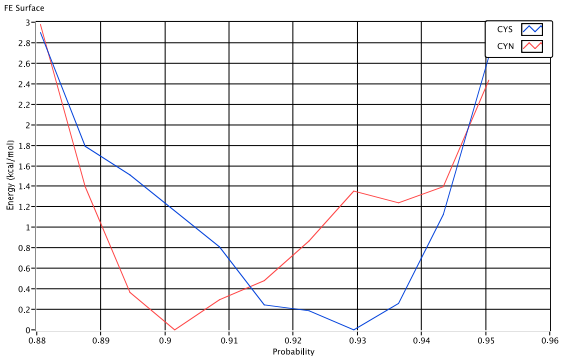
Pairwise contacts are considered native when they occur more than 2/3 of the trajectory. In the heavy atom analysis uses a cutoff distance of 4 Angstroms, and interactions within **two** atoms along the backbone are omitted. This parameter might need to be changed in favor of removing more backbone interactions that can distort the native contact precentages.

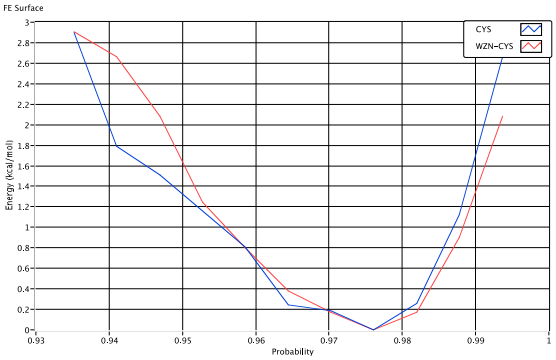
These plots show the Free energy surface based on the probabilities of native contacts, compared to the biologically relevant bound zinc case. The y-axis should be Enerfy (kcal/mol). First, we have the No zinc deprotonated cysteines….I realize this is not consistent with the legend-don't trust the legend. Then the two cases with zinc, followed up by the other biologically relevant case. The well is shifted to a lower probability in all three cases, suggesting that the bound zinc motif is favorable.

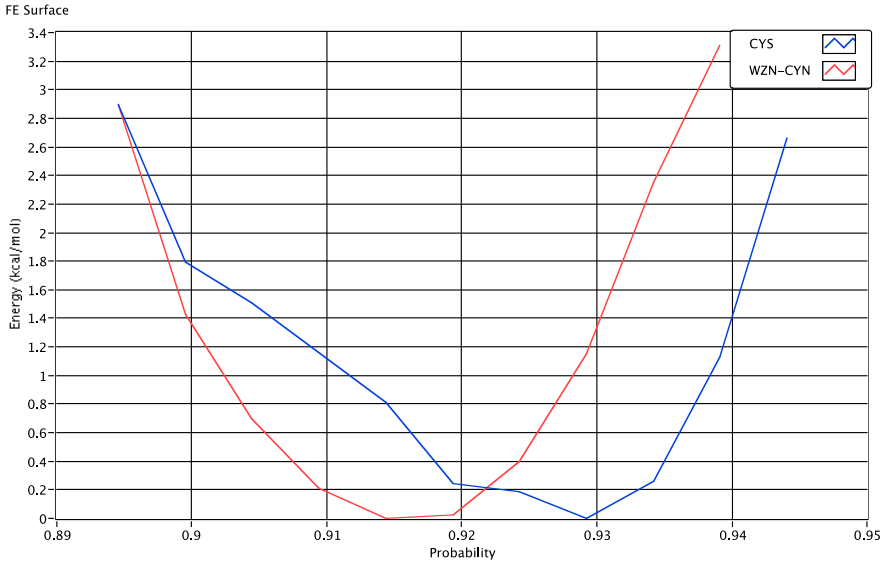




This analysis is also interesting if we look at the other biologically relevant case of no Zinc and protonated cysteines.







Poisson Boltzmann Analysis

While this has yet to be analyzed for the microsecond sims, there are some results worth noting at the 15ns regime. …

Local Unfolding

In the biologically bound zinc case (WZN-CYN) there is noticeable local unfolding characterized by motion of the last binding cysteine (residue 26 of 28). The working hypothesis here is that this local unfolding is part of a search mechanism for other binding moieties whether that be another zinc finger or possibly a TPEN molecule

For the No Zinc CYS case, we see much larger portions of the protein unfolding. Again see pdf slides for image details.

Supplemental

A great deal of the analysis presented in this document was analyzed using code developed to automate the analysis of molecular dynamics simulations. Currently, efforts are underway to automate the setting up and execution of the simulations with the larger aim to automate the whole process from generating the protein environment from a particular pdb (or set of input) through the analysis, and incorporating an iterative process where the code can setup and execute further simulations based on the analysis of the previous run. This project will take sometime, but could provide us theorists with a high-throughput, objective way of generating and analyzing lots and lots of data.